PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/12, A61K 48/00
C07K 13/00, C12N 15/86

A1

(11) International Publication Number: WO 94/06910

(43) International Publication Date: 31 March 1994 (31.03.94)

(21) International Application Number: PCT/US93/08844

(22) International Filing Date: 17 September 1993 (17.09.93)

(30) Priority data:
07/948,289 18 September 1992 (18.09.92) US

(60) Parent Application or Grant
(63) Related by Continuation
US 07/948,289 (CIP)
Filed on 18 September 1992 (18.09.92)

(71) Applicant *(for all designated States except US)*: CANJI, INC. [US/US]; 3030 Science Park Road, Suite 302, San Diego, CA 92121-0177 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : SHEPARD, H., Michael [US/US]; 2745 Argonauta Street, La Costa, CA 92009 (US). KAN, Nancy [US/US]; 4417 Camden Circle, Dublin, OH 43017 (US).

(74) Agents: KONSKI, Antoinette, F. et al.; Campbell & Flores, 4370 La Jolla Village Drive, Suite 700, San Diego, CA 92122 (US).

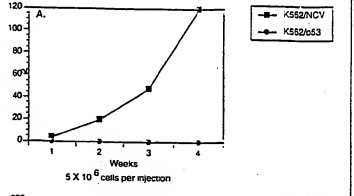
(81) Designated States: AU, CA, JP, KR, NO, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

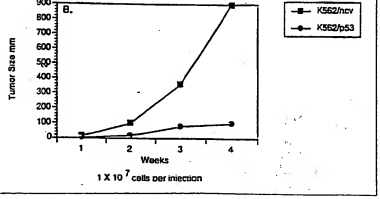
Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GENE THERAPY BY RETROVIRAL VECTOR WITH TUMOR SUPPRESSIVE GENE

(57) Abstract

A method for transducing a pathologic hyperproliferative mammalian cell is provided by this invention. This method requires contacting the cell with a suitable retroviral vector containing a nucleic acid encoding a gene product having a tumor suppressive function. Also provided by this invention is a method for treating a pathology in a subject caused by the absence of, or the presence of a pathologically mutated tumor suppressor gene.





GENE THERAPY BY RETROVIRAL VECTOR WITH TUMOR SUPPRESSIVE GENE.

FIELD OF THE INVENTION

The present invention generally relates to a method for selectively transducing pathologic hyperproliferative mammalian cells in a heterogeneous cell preparation comprising retroviral-mediated transduction of the pathologic cell with a nucleic acid encoding a gene product having tumor suppressive function.

Throughout this application various publications are referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

The human p53 gene encodes a 53 kilodalton nuclear phosphoprotein (Lane, D.P., et al., Genes and Dev., 4:1-8 (1990); Lee, Y-HP, Breast Cancer Res.and Trmt, 19:3-13 (1991); Rotter, V., et al., Adv. Can. Res., 57:257-72 (1991)). The p53 protein was first identified as a cellular protein in SV40-transformed cells that was tightly bound to the SV40 T antigen (Lane, D.P., et al. Nature, 278:261-3 (1979)). The wild type p53 gene has the characteristics of a tumor suppressor gene. It is similar to the prototype of tumor suppressor genes, the retinoblastoma gene (RB), in that loss of heterozygosity of the p53 or RB genes characterizes the phenotype of many types of tumor cells (Hollstein, M. et al., Science, 253:49-51 (1991); Levine, A.J., et al., Biochimica et Biophysica Acta, 1032:119-36 (1990); Levine, A.J., et al., Nature 351:453-6 (1991); Weinberg, R.A. Science, 254:1138-46 (1991)). In human malignancies associated with p53

type p53 can reverse the malignant phenotype as measured by colony formation in soft agar and tumor formation in nude mice (Chen, P.L., et al., Science, 250:1576-80 (1990); Cheng, J., et al., Can. Res., 52:222-6 (1992); Baker, S.J., et al., Science, 249:912-15 (1990); Isaacs, W.B., et al., Can. Res., 51:4716-20 (1991); Casey, G., et al., Oncogene, 6(10):1791-7 (1991); Shaw, P., et al., PNAS USA, 89:4495-99 (1992); Takahashi, T., et al., Can. Res., 52:2340-3 (1992)). Tumor cell types which have shown conversion of a non-malignant phenotype as a result of the introduction of wild type p53 expression include prostate (Isaacs, W.B., et al., supra) , breast (Casey, G. et al. supra), colon (Baker, SJ., et al., supra; Shaw, P. et al., supra) lung (Takahashi, T. et al., supra) , and lymphoblastic leukemia (Cheng, J. et al., supra). Other data suggest that introduction of wild type p53 into tumor cells which have lost endogenous p53 expression appears to be cytotoxic (Johnson, P. et al., Mol. Cell. Biol., 11(1):1-11 (1991)). In some cases the re-introduction of wild type p53 may result in programmed cell death, or apoptosis (Yonish-Rouach, E. et al., Nature, 352:345-7 (1991)). The work described above indicates strongly that alteration of the wild type p53 gene has a role in multiple aspects of tumorigenesis and that reintroduction of the wild type p53 coding sequence can have a negative regulatory function or cytotoxic effect on malignant cells.

Clinical data suggest that inactivating mutations in the p53 gene are among the most common types of mutations associated with human malignancy (Rotter, V. et al. supra; Nigro, J.M. et al., Nature, 342:705-8 (1989); Gaidano, G. et al., PNAS USA, 88:5413-7 (1991); Cheng, J. et al., Mol. Cell. Biol., 10(10):5502-09 (1990)). A classical example is the Li-Fraumeni syndrome, a familial syndrome of several neoplasms, including breast cancer, sarcomas and others. Specific mutations in the p53 gene are found in affected members of the family and appear to

introduction of a stably expressed tumor suppressor gene into a heterogeneous cell preparation (containing both normal and pathologic hyperproliferative cells) and, under suitable conditions, selectively transducing phenotypically pathologic hyperproliferative cells, suppressing the pathologic phenotype and reinfusing the treated cell preparation into the patient. Also provided by this invention is a method for treating a pathology in a subject caused by the absence of, or the presence of a pathologically mutated tumor suppressor gene.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the tumorigenicity of antibiotics-selected K562 cells in nude mice. K562 cells were infected with the p53-RV or NCV and selected in hygromycin as described in the legend to Table 3. (A) $5X10^6$ K562/p53 or K562/NCV (B) $1X10^7$ K562/p53 or K562/NCV were injected subcutaneously into opposite flanks of athymic Balb/c nu/nu mice. The mice were purchased from Simonsen Laboratories, Inc. (Gilroy, CA) and maintained in a pathogen-free environment. Once tumors were formed, they were measured weekly until the experiments were terminated.

Figure 2 shows delayed tumor formation in nude mice induced by K562 cells following a short-term infection with the p53-RV. K562 cells were infected with the p53-RV or NCV for 4 hours as described in Example II. The viral supernatant was removed and the cells were injected immediately into nude mice as described in Example III.

Figure 3 shows delayed tumor formation in nude mice induced by three human cell lines following short-term infections with the p53-RV. The three human cells lines, H69 (human small-cell lung carcinoma), H128 (human small-cell lung carcinoma) and HTB9 (human bladder carcinoma),

Trtmt., 20:511-14 (1991)). Approximately 30% of these patients are candidates for tumor suppressive gene therapy. This number derives from the observation that about 30% of cancer patients either do not express the tumor suppressor gene or express an inactivated form of the tumor suppressor protein (Hollstein, M. et al., supra) . The preferred embodiments detailed below support the efficaciousness of a retrovirus encoding the human wild type tumor suppressor gene, p53-RV, in reversing the malignant phenotype of several leukemia and lymphoma cell lines as measured by abrogation or substantial inhibition of colony formation in soft agar assays, and as judged by reversing/inhibiting the ability of tumor cells to grow in nude mice following introduction of the wild type p53 gene.

For the K562 tumor cell line, which is derived from a chronic myelogenous leukemia patient in blast crisis (Andersson, L.C. et al., Int. J. Can., 23:143-7 (1979)) for two human small-cell lung carcinoma cell lines (H69 and H128) (Gazdar, A.F. et al,. Can. Res., 40(10):3502-7 (1980)), and for one transitional cell (bladder) carcinoma cell line (HTB9) (Takahashi, R. et al., PNAS USA, 88:5257-61 (1991)) tumor suppression by p53 can be accomplished with a protocol involving short-term infections with the p53-RV. This protocol is completely consistent with current clinical methodology used in the preparation of bone marrow or peripheral blood hematopoietic cells for autologous bone marrow transplantation (ABMT) (Deisseroth, A.B. et al., Human Gene Therapy, 2:359-376 (1991)).

The present invention generally relates to an improved method of gene therapy for "negative purging" of pathologic hyperproliferative cells that contaminate preparations of autologous hematopoietic cells used for bone marrow reconstitution. As used herein, the term "hyperproliferative cells" includes but is not limited to cells having the capacity for autonomous growth, i.e.,

suppressor gene into the cell preparation (whether derived from autologous peripheral blood or bone marrow). As used herein, a "suitable sample" is defined as a heterogeneous cell preparation obtained from a patient, e.g., a mixed population of cells containing both phenotypically normal and pathogenic cells. An example of a wild type tumor suppressor gene is the p53 gene, the coding sequence has been described by Chen et al. supra and is shown in Table 1.

TABLE 1

```
                                                  50
V*SHR PGSR* LLGSG DTLRS GWERA FHDGD TLPWI GSQTA FRVTA MEEPQ
                                                 100
SDPSV EPPLS QETFS DLWKL LPENN VLSPL PSQAM DDLML SPDDI EQWFT
                                                 150
EDPGP DEAPR MPEAA PPVAP APAAP TPAAP APAPS WPLSS SVPSQ KTYQG
                                                 200
SYGFR LGFLH SGTAK SVTCT YSPAL NKMFC QLAKT CPVQL WVDST PPPGT
                                                 250
RVRAM AIYKQ SQHMT EVVRR CPHHE RCSDS DGLAP PQHLI RVEGN LRVEY
                                                 300
LDDRN TFRHS VVVPY EPPEV GSDCT TIHYN YMCNS SCMGG MNRRP ILTII
                                                 350
TLEDS SGNLL GRNSF EVRVC ACPGR DRRTE EENLR KKGEP HHELP PGSTK
                                                 400
RALPN NTSSS PQPKK KPLDG EYFTL QIRGR ERFEM FRELN EALEL KDAQA

GKEPG GSRAH SSHLK SKKGQ STSRH KKLMF KTEGP DSD*
```

* = Stop codon

The preferred delivery system for the wild type tumor suppressor gene is a replication-incompetent retroviral vector. As used herein, the term "retroviral" includes, but is not limited to, a vector or delivery vehicle having the ability to selectively target and introduce the coding sequence into dividing cells. As used

consuming procedure of transducing cell samples with a selectable marker gene, such as neomycin, to identify pathologic cells to facilitate subsequent attempts to remove those cells before reinfusion into the patient.

Other vectors are suitable for use in this invention and will be selected for effecient delivery of the nucleic acid encoding the tumor suppressor gene. The nucleic acid can be DNA, cDNA or RNA.

The subject invention provides a "shotgun" procedure whereby the cell sample is contacted with a retroviral vector in the absence of selective medium that does not necessarily contain a selectable marker gene, but notwithstanding, possesses the ability to simultaneously selectively target and transduce only the pathologic cell population in the heterogeneous cell preparation. Other methods of efficient delivery or insertion of a gene of interest into a cell are well known to those of skill in the art and comprise various molecular cloning techniques. As used herein, the terms "insertion or delivery" encompass methods of introducing an exogenous nucleic acid molecule into a cell.

A variety of techniques have been employed in an attempt to deplete marrow of pathologic hyperproliferative cells before reinfusion, utilizing "purging" methods, e.g., monoclonal antibodies or chemotoxins (Kaizer H. et al., Blood, 65:1504 (1985); Gorin, N.C. et al., Blood, 67:1367 (1986); De Fabritiis, P. et al., Bone Marrow Transplant, 4:669 (1989)). As used herein, the term "pathologic" includes abnormalities and malignancies induced by mutations and failures in the genetic regulatory mechanisms that govern normal differentiation that are not the result of gene loss or mutation. These techniques, however, have not resulted in reduced relapse rates, and have consistently resulted in damaging normal marrow progenitor

that the cell is transduced. In one embodiment, the gene product is expressed by a tumor suppressor gene and the tumor suppressor gene can be, but is not limited to wild type p53 gene, retinoblastoma gene RB, Wilm's tumor gene WT1 or colon carcinoma gene DCC. Additionally, the nucleic acid is DNA, cDNA or RNA.

The suitable conditions for contacting can be by infecting the sample cells in the absence of selective medium. "Suitable retroviral vector" has been defined above. This method is particularly useful when the pathological cells being contacted are prostate cells, psoriatic cells, thyroid cells, breast cells, colon cells, lung cells, sarcoma cells, leukemic cells or lymphoma cells.

The suitable time period for contacting can be less than about ten hours, or more specifically, about four hours. Transduction can be known to be complete, for example, when the hyperproliferative phenotype is characterized by the transduced cell expressing a mature or benign phenotype or by apoptosis or death of the transduced cell. This method has been shown to reduce tumor formation or tumorigenicity in a subject.

This method can be practiced ex vivo or in vivo. The practice of the ex vivo method is described above. When the method is practiced in vivo, the retroviral vector can be added to a pharmaceutically acceptable carrier and systemically administered to the subject. In one embodiment, the subject is a mammal, such as a human patient. Acceptable "pharmaceutical carriers" are well known to those of skill in the art and can include, but not be limited to any of the standard pharmaceutical carriers, such as phosphate buffered saline, water and emulsions, such as oil/water emulsions and various types of wetting agents.

resulted in either a reduction or elimination of colony formation in soft agar.

TABLE 3

Cell Line	No. of Cells Seeded	Plating Efficiency
HL-60	$5X10^5$	TMTC
HL-60/T*	$5X10^5$	4.7%
HL-60	10^5	43%
HL-60/T*	10^5	0%
HL-60	$5X10^4$	55%
HL-60/T*	$5X10^4$	0%
Hut 78	10^5	9.4%
Hut 78/I#	10^5	0.39%
Hut 78	$5X10^4$	9.2%
Hut 78/I#	$5X10^4$	0%
Molt 3	10^5	11.7%
Molt 3/I#	10^5	1.5%

*Transfected
#Infected

The human leukemic cell lines, HL-60, Hut 78 and Molt 3, were obtained from American Type Culture Collection (ATCC). The cell lines Hut 78 and Molt 3 were infected with the p53-RV and the HL-60 cell line was transfected with p53-RV DNA. The p53-RV containing the wild type p53 cDNA isolated from human fetal brain and Moloney murine leukemia viral vector has been described by Chen et al., supra. This virus also carries the hygromycin resistant gene whose expression is driven by the Rous sarcoma virus (RSV) promoter sequence. The murine NIH3T3-derived packaging cell line, PA12 (Chen et al., supra), produces the p53-RV with titers ranging from 1×10^5 to 1×10^6 virus per ml.

cells. The plating efficiencies of the NCV-infected K562 cells were similar at all three multiplicities of infection. The latter result suggests that the does-dependent reduction in tumor cell colony formation observed with increasing doses of p53-RV was due to introduction of the wild type p53. Furthermore, the result with NCV indicates that there is little non-specific toxicity associated with the retroviral infection up to MOI of 10, as measured by this assay.

TABLE 4

Virus Infection	No. of Cells Seeded	M.O.I.	Plating Efficiency (Colony No.)
p53-RV	10^4	1	3.10% (310)
		3	0.52% (52)
		10	0% (0)
Control RV	10^4	1	4.30% (430)
		3	5.30% (530)
		10	3.40% (340)
p53-RV	$5X10^3$	1	4.40% (220)
		3	1.80% (90)
		10	0.25% (13)
Control RV	$5X10^3$	1	4.70% (235)
		3	4.10% (205)
		10	6.50% (325)

Human chronic myelogenous leukemia (CML)-derived cell line, K562, was obtained from ATCC, Accession No. CCL243. To perform the short-term infections, K562 cells were infected with the p53-RV or NCV for 4 hours as described in Example I. Multiplicity of infection (MOI) was determined from the titer of the viral stocks and K562 cell number. At the end of infection, the viral supernatant was removed by pelleting the cells, and the concentrated cells were used immediately in the soft-agar assay as described in Example I.

of the wild type p53 gene in K562 cells, and to determine whether a short-term infection protocol would be feasible for potential therapy of leukemias and lymphomas, K562 CML cells were co-incubated with p53-RV for four hours before testing for the malignant phenotype as determined by subcutaneous tumor formation in nude mice. Following a short-term infection by the p53-RV or the NCV, K562 cells were injected bilaterally into nude mice. In three separate experiments, substantial suppression of tumor formation on the flank injected with K562 exposed to the p53-RV was observed (Fig. 2).

EXAMPLE V

Growth suppressive activity of p53-RV on other human tumor cell types

While the major target for clinical trials consists of leukemia and lymphoma patients, other cancer patients are currently under consideration for clinical trials involving marrow reconstitution (Miller, C.W. et al., Can. Res., 52:1695-8 (1992); Takahashi, T. et al., Oncogene, 6:1775-8 (1991); Takahashi, T. et al., Science,491-4 (1989)). Figure 3 demonstrates that short-term infections of two small-cell lung carcinoma cell lines (H69 in Fig. 3A; H128 in Fig. 3B) lead to substantial inhibition of tumor growth in nude mice. In addition, a similar experiment was performed with a human transitional cell (bladder) carcinoma cell line (HTB-9 in Fig. 3C). In contrast, tumor cells infected with NCV grow rapidly in this tumor model (Fig. 3A-C).

TABLE 5
COLONY NUMBER

Infection	<----------rHuGMCSF----------> None	0.02ng/ml	0.04ng/ml
Control	1	18	21
p53-RV(0.1)	0	16	28
NCV(0.1)	0	11	18
MOCK(0.1)	2	12	17
p53-RV(1.0)	2	15	23
NCV(1.0)	4	9	18
MOCK(1.00)	0	25	18

EXAMPLE VII

Negative Purging of Small Cell Lung Cancer Cells (H69) From a Preparation of Human Bone Marrow

Increasing quantities of small-cell lung cancer cell line H69 were added to human bone marrow cells. These cells were subjected to 3 two hour cycles of infection with p53-RV at a M.O.I. of 3. After infection the cells were pelleted and plated in methylcellulose. Colony formation is shown in Table 6. Suppression of tumor cell colony formation is evidenced in the p53-RV treated cultures, but is absent in the mock infected cultures. There is no evidence of suppression of bone marrow colony formation units in either case.

cells and 3 x 10^6 uninfected cells. The ratio of infected to uninfected cells = 1:2. Every mouse in group 4 was injected subcutaneously with a mixture of 0.45 x 10^6 infected and 4.5 x 10^6 uninfected cells (infected:uninfected = 1:10). All the mice in group 5 were injected subcutaneously with 5 x 10^6 uninfected cells (infected:uninfected = 0:1). Nude mice were observed for tumor growth and survival time. Results of the study are summarized below.

Group	Ratio infected:uninfected	Tumor Formation	Survival Status[b]
1	1:0	-	Alive and healthy at 200 days
2	1:1	-	Alive and healthy at 200 days
3	1:2	-	Alive and healthy at 200 days
4	1:10	-	Alive and healthy at 200 days
5	0:1	+[a]	All died within 90 days

a Measurable tumors developed by thirtieth day.

b Experiment was terminated on day 200, when all p53RV-selected animals were till alive and healthy.

WE CLAIM:

1. A method for transducing a pathologic hyperproliferative mammalian cell comprising contacting the cell with a suitable retroviral vector containing a nucleic acid encoding a gene product having a tumor suppressive function, under suitable conditions such that the cell is transduced.

2. The method of claim 1, wherein the gene product is expressed by a tumor suppressor gene.

3. The method of claim 2, wherein the tumor suppressor gene is wild type p53 gene, retinoblastoma gene RB, Wilm's tumor gene WT1 or colon carcinoma gene DCC.

4. The method of claim 1, wherein the suitable conditions are infecting the sample cells in the absence of selective medium.

5. The method of claim 1, wherein the suitable retroviral vector lacks a selectable marker gene.

6. The method of claim 1, wherein the suitable retroviral vector is replication-incompetent.

7. The method of claim 1, wherein the pathological cells are prostate cells, psoriatic cells, thyroid cells, breast cells, colon cells, lung cells, sarcoma cells, leukemic cells or lymphoma cells.

8. The method of claim 1, wherein the suitable time period is less than about ten hours.

9. The method of claim 8, wherein the time period is about four hours.

20. The method of claim 16, wherein the absence or presence of a pathologically mutated tumor suppressor gene causes a cell to hyperproliferate.

21. The method of claim 20, wherein the hyperproliferative cell is a prostate cell, a psoriatic cell, a thyroid cell, a breast cell, a colon cell, a lung cell, a sarcoma cell, a leukemic cell or a lymphoma cell.

22. The method of claim 21, wherein the treating of the hyperproliferative cell is characterized by apoptosis or death of the cell.

23. The method of claim 16, wherein the contacting is effected in vivo.

24. The method of claim 16, wherein the nucleic acid is RNA.

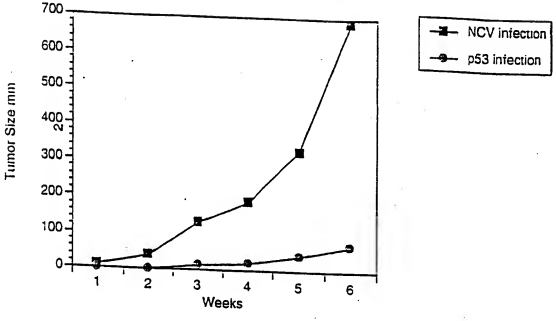
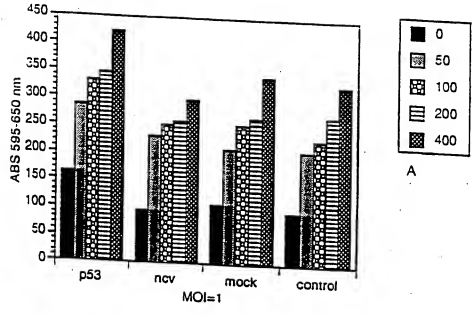

700
600
500
400
300
200
100
0
Tumor Size mm
1
2
3
4
5
6
Weeks
NCV infection
p53 infection

FIGURE 2


450
400
350
300
250
200
150
100
50
0
ABS 595-650 nm
p53
ncv
mock
control
MOI=1
0
50
100
200
400
A

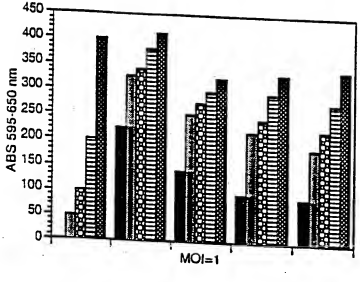

450
400
350
300
250
200
150
100
50
0
ABS 595-650 nm
MOI=1
B.

FIGURE 4

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US 93/08844

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 13, 16 - 24 and partially 1-12 and 14,15 as far as they concern an in vivo method of treatment of the human/ animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.